RESEARCH PLAN PROPOSAL

COMPARATIVE NEUROTOXIC EFFECTS OF FLOURIDE AND ALUMINIUM IN MALE SWISS ALBINO MICE.

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Submitted by

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INTRODUCTION:

Fluorine is one of the most abundant and widely distributed elements in nature. It is desirable in very limited quantities for healthy growth of bones and teeth preventing dental cavities, but in excess causes a disease known as fluorosis. Other deleterious effects of excess fluoride on human health are: damage to kidney tubules due to continuous filtration of the ingested fluoride, dysfunction of reproductive system and ageing (Sharma *et al,*2007).

In India, fluorosis is endemic in 17 states including Rajasthan due to excess of fluoride content in the groundwater. Various anthropogenic sources contributing fluoride are steel, aluminium, ceramics and phosphate fertiliser production, as well as coal combustion (Smith and Hedge 1979; Hillier *et al*,2000). The permissible limits of fluoride in drinking water as suggested by Bureau of Indian Standards vary between 0.6 to 1.2 ppm and World Health organization permits a maximum of 1.5 ppm. Chronic exposure to fluoride above the permissible limits leads to harmful effects on different tissues and metabolic processes.

Aluminium is the third most abundant metal on earth and reports have revealed that it is a major neurotoxin and disrupter of neurological function (Blaylock, 2012). Aluminium is the most widely distributed metal on the planet and it is used in the production of many every-day products. Cookware is made from aluminium, soda cans are aluminium, and aluminium foil is found in most kitchens. Aluminium is also in antacids, aspirin, vaccines, and even flour. Unlike vitamins, minerals, and trace elements, the body does not need aluminium. Aluminium accumulates in the kidneys, brain, lungs, liver and thyroid where it competes with calcium for absorption and can affect skeletal mineralization. In infants, this can slow growth. Exposure to aluminium, unfortunately, is common with some occupations like mining, factory work, and welding. Welding can be especially worrisome because it produces vapours that, when inhaled, send aluminium directly into the lungs from where it is released to the blood and distributed to the bones and brain. Pesticides exposure through pesticides containing aluminium like aluminium phosphide has also caused serious problems in farmers (Wikipedia).

SOURCES OFALUMINIUM AND FLOURIDE INTERACTION:

1) THROUGH DRINKING WATER: Aluminium has a strong affinity for fluoride, and therefore is used in fluoride-affected areas for defluoridation of potable water by the Nalgonda and Activated Alumina techniques. The problem associated with these techniques is higher residual aluminium concentrations in the defluoridated water (Nalgonda Process = 2.01 - 6.86 ppm; Activated Alumina technique = 0.15 - 0.45 ppm), which exceed the permissible limit (0.2 ppm) for drinking water (Gupta 1997). Alum application for removal of suspended impurities by Public Health Engineering Departments also contributes residual aluminium to the drinking water supply, especially in soft water.

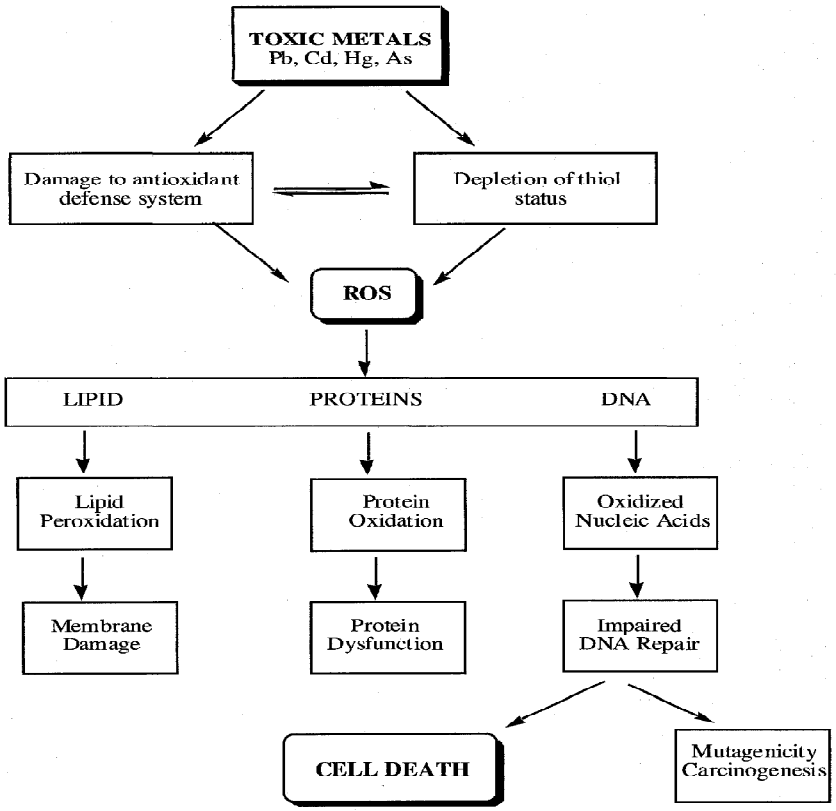
2) THROUGH GROUNDWATER: Pesticides containing aluminium leach into the soil and interact with fluoride present in groundwater so the chances of receiving aluminium fluoride combination through water increases in areas with high groundwater fluoride concentration [Gurjar *et al* (2011)].

3) OTHER SOURCES: Aluminium metal is used as a structural material in the construction, automotive and aircraft industries, in the production of metal alloys, in the electric industry, in cooking utensils, and in food packaging. Aluminium compounds are used as antacids, antiperspirants, and food additives (ATSDR, 1992). Aluminium salts are also widely used in water treatment as coagulants to reduce organic matter, colour, turbidity, and microorganism level. Foods naturally high in aluminium include potatoes, spinach, and tea. Processed dairy products, flour, and infant formula may be high in aluminium if they contain aluminium-based food additives (FAO/WHO, 1989; Pennington & Schoen, 1995; WHO, 1997).After entering through various routes aluminium and fluoride can form toxic complexes inside the body.

BRAIN AND OXIDATIVE DAMAGE:

One of the major toxic effects which most of metals cause is Oxidative stress in different tissues. Organisms under oxidative stress are capable of inducing antioxidant enzymes (Venkataiah,1995). When the production of reactive oxygen species exceeds the ability of the antioxidant system, it results in oxidative stress due to high levels of reactive oxygen species. Brain is prone to oxidative stress due to presence of high levels of polyunsaturated fatty acids, relatively low antioxidant capacity, presence of redox metal ions like iron and copper and high oxygen (Madhusudhan *et al*, 2010). Damage due to free radicals caused by ROS leads to several damaging effects as they can attack lipids, protein/ enzymes, carbohydrates, and DNA in cells and tissues. They induce undesirable oxidation, causing membrane damage, protein modification, DNA damage, and cell death induced by DNA fragmentation and lipid peroxidation(Singh *et al* ,2004) .The oxidative-stress state has an important role in the development of many degenerative diseases, such as autoimmune disease, cancer, cardiac disease, and diabetes, but it also has a crucial role in the neurodegenerative diseases, such as Alzheimer's (Pan *et al*,2011),Parkinson's (Sevcsik *et al*,2011). "The pathological changes found in the brain tissue of the animals given fluoride and aluminium-fluoride were similar to the alterations found in the brains of people with Alzheimer's disease and other forms of dementia," reads an announcement associated with the publishing of the *Brain Research* study.

MECHANISM OF METAL INDUCED TOXICITY: [Ercal *et al*(2001)]



REVIEW OF LITERATURE:
FLOURIDE:

Vani and Reddy(2000) found that when mice treated with NaF (20 mg/kg body mass) for 14 days shows decreased SOD, CAT, and glutathione transferase (GST) activities in brain.

Tao *et al*(2005) observed that when sodium fluoride was administered in increasing doses i.e. 50 mg/kg, 100 mg/kg and 150 mg/kg to 3 groups of pigs respectively,the contents of Malondialdehyde (MDA)increased and total antioxidant capacity (T-AOC) levels, activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), glutathione transferase (GST) and xanthine oxidase (XOD) decreased. This indicated that excess of

fluoride causes impairment of soft tissues involved in lipid peroxidation and decreases the activities of some enzymes associated with free radical metabolism.

Bhatnagar *et al* (2006) observed that when swiss albino mice were given sodium fluoride in drinking water, activities of Acetylcholinesterase and Butrylcholinesterase showed a concentration-dependent decrease in brain. Superoxide dismutase (SOD) and catalase (CAT) also showed a significant concentration dependent decrease in brain. Malondialdehyde (MDA) levels showed an initial decrease at 60 ppm flouride that changed to a significant increase at 120 ppm. Ascorbic acid exhibited concentration dependent increase whereas total protein showed a concentration dependent decrease in brain.

High-level exposure can allow excessive amounts of flourine to penetrate the blood brain barrier in rats and cause neuronal degeneration by a combination of events that may impair normal functioning (Madhusudan *et al*, 2009).

Madhusudhan *et al* (2010) found that when female rats were treated with flouride during their pregnancy period, pups born to them showed increase in the concentration of malondialdehyde (MDA) in all the regions of the brain, indicating intensified LPO. It also resulted in decreased activity of CAT, SOD, and GSH-Px in discrete regions, indicating the suppressed antioxidant efficiency in combating the F induced free radical damage.

Sodium fluoride treatment in rat resulted in axon deterioration, myelin sheath degeneration and presence of dark cells with scanty cytoplasm in spinal cord and sciatic nerve. NaF treatment also caused other distinctive morphological alterations like vacuolated swollen mitochondria in neocortex, hippocampus and cerebellum, myelinated fibers with breaks in continuity (axon partly preserved and partly vacuolated) in hippocampus; myelin splitting in cerebellum and vacuolated schwann cell in sciatic nerve . Neurodegeneration was clearly evident in the hippocampus, neocortex, cerebellum, spinal cord and sciatic nerve on sodium fluoride exposure brain (Reddy *et al*,2011).

ALUMINIUM:

Half life of aluminium elimination in the brain is estimated at >100 days which is a reason for the neurodegeneration caused by aluminium (Greger *et al*, 1997).

Aluminium is a neurotoxin at concentrations as low as 0.08 ppm (Hewitt *et al*,1990) and also affects haematology (Turgut *et al*, 2007), kidney, liver (Stacchiotti *et al*, 2006) and intestinal flora (Yan *et al*, 1998).

Vander *et al*(1999) cultured cells of the hippocampus obtained from rat foetuses when exposed to AlF_3 alone, resulted in the formation of abnormal connections between nerve fibers . This effect was increased when AlF_3 was combined with NaF.

The elimination of aluminium from the body is very slow. The half life for total body aluminium has been estimated at 7 years in humans and reflects redistribution from bone stores (Yokel *et al*, 2001).

Sushma *et al*(2009) concluded that aluminium acetate administration in mice causes a decrease in SOD and catalase in a dose dependent manner. Non-significant change in SOD was recorded. There was an increase in malondialdehyde formation or lipid peroxidation with increase in dose in brain.

Yuan *et al*(2012) found that there was a increase in lipid peroxidative products (TBARs), catalase activity and glutathione peroxidase activity and decrease in SOD activity in hippocampus, diencephalon, cerebellum, and brain stem of rats treated with high aluminium chloride.

When rats were exposed to aluminium chloride for a long period the levels of lipid peroxidation were significantly increased, while the activities of superoxide dismutase (SOD) as well as reduced glutathione (GSH) content were significantly decreased in the brains of rats. Acetylcholinesterase activity also showed a significant increase (Naidu *et al*,2013).

Aluminium chloride administration significantly decreased the level of GSH and the activities of SOD, CAT, GPx, Na+/K+ ATPase, Ca2+ ATPase and Mg2+ ATPase and increased the level of LPO and the activities of ALP, ACP, ALT and AST in all the brain regions when compared with control rats (Thangarajan *et al* ,2013).

ALUMINIUM AND FLUORIDE COMBINATION:

Varner *et al*(1994) observed that binding of aluminium with fluoride may increase aluminium bioavailability to humans from drinking water.

Simultaneous administration of fluorine and aluminium in drinking water increased plasma concentrations of aluminium in rats. [Allain *et al(1996)]*

Glynn *et al(1999)*reported that on uptake of aluminium in rats forms soluble complexes with citrate and fluoride, such as aluminium citrate(97%) and aluminium fluoride (60%).

Kaur *et al*(2009) observed that when rats were exposed to fluoride, there was an increase in LPO levels and decrease in SOD activity in different regions of brain like the cerebrum, cerebellum, and medulla oblongata. The neurotransmitter (e.g., dopamine, nor epinephrine, and serotonin) content was also altered. These aspects were more pronounced in animals given fluoride and aluminium together.

Combination of fluoride and aluminium in rat brain resulted in appearance of vacuolar spaces, cellular fragmentation and ghost-like appearance of neuronal cells of the treatment group, while the cells of the control group appeared intact with regular cell morphology and intact cellular integrity (Akinrinade *et al*,2013).

Aluminium, fluoride and a combination of aluminium–fluoride treatments caused an increase in brain lipid per oxidation products and reactive oxygen species (ROS) formation. An increase in glial activation and inflammatory response were seen in treated groups of rats versus the control (Akinrinade *et al* ,2014).

OBJECTIVES:

1) Determine the level of oxidative stress induced by flouride and aluminium alone and in combination in brain.
2) Interaction of fluoride and aluminium: Synergistic or Antagonistic.
3) Trace the pathway of Neurodegeneration due to oxidative stress: DNA damage and Apoptosis.
4) Possible reversal of toxicity by some natural product.

MATERIALS AND METHODS:

EXPERIMENTAL ANIMAL: Swiss male albino mice of age 8-10 weeks will be used. The animals will be housed under standard laboratory conditions and maintained on natural light and dark cycle and will have free access to food and water. Animals will be adapted to laboratory conditions before the experiment. Each group will have minimum six animals at the time of autopsy.

METALS:

Flouride will be given in form of Sodium Flouride (NaF)
Aluminium will be given in form of Aluminium Chloride($AlCl_3$).
Combination of Aluminium Chloride and Sodium Fluoride.

DOSE:

20% of LD50 dose will be used. [Chinoy *et al*(2000)]

- Sodium fluoride:10 mg/kg body weight
- Aluminium chloride: 200 mg/kg body weight
- Sodium fluoride+ Aluminium chloride: 5mg/kg bw+ 100 mg/kg bw
- A higher dose and a lower dose from the 20% LD50 values may be taken to further support the results.

MODE OF ADMINISTRATION OF DOSE:

Treatment will be given daily orally using hypodermic Syringe attached to angular needle.

GROUPS OF EXPERIMENTAL SUBJECT:

Animals will be divided into 6 groups, each group having 6 male swiss albino mice.

Duration of treatment: 60 days.

GROUP	DOSE	1^{ST} AUTOPSY	2^{ND} AUTOPSY	3^{RD} AUTOPSY
I	CONTROL- Distilled Water	31DAY	46 DAY	61 DAY
II	NaF TREATED	31 DAY	46 DAY	61 DAY
III	$AlCl_3$ TREATED	31 DAY	46 DAY	61 DAY
IV	NaF+$AlCl_3$ TREATED	31DAY	46 DAY	61 DAY
V	NaF+$AlCl_3$ treated then withdrawal for one month	75DAY	90 DAY	
VI	NaF+$AlCl_3$+ Natural Product	31 DAY	46 DAY	61 DAY

In group V the animals after treatment for 60 days will be left for recovery for one month (30 days). The autopsy will be done on 75^{th} and 90^{th} day.

PARAMETERS :

All the parameters will be performed on each autopsy interval in different groups.

1) Coefficient of brain to body weight: Ratio of weight of brain/ body weight
2) Histopathology of brain
3) Oxidative stress parameters: Antioxidants
   i) Superoxide Dismutase (SOD)(Kono *et al* 1978)
   ii) Catalase (Sinha *et al* 1972)
   iii) Glutathione peroxidise (Rotruck *et al* 1973)
   vi) Lipid Peroxidation : MDA (Ohkawa *et al* 1979)
   vii) Glutathione (GSH) (Ellman *et al* 1959)
4) DNA Damage
5) Apoptosis

STATISTICAL ANALYSIS:

The data will be expressed as Mean ± S.E. (Standard error). The statistical significance between groups will be analyzed using 't' test or ANOVA.

SIGNIFICANCE:

- The possible mechanism of oxidative stress caused by combination of fluoride and aluminium can be traced.
- Our study can contribute in determining the role and mechanism of aluminium and fluoride in neurodegenerative disorders.
- A comparative account of toxicity induced by aluminium, fluoride and their combination can be determined in terms of oxidative stress, DNA damage and Apoptosis. This will provide an idea whether aluminium and flouride interaction is synergistic or antagonistic.
- Our study will add to the knowledge of toxicity induced by multiple metal exposures.

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